

The Intracellular Localization of the Vanillin Biosynthetic Machinery in Pods of *Vanilla planifolia*

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Vanillin is the most important flavor compound in the vanilla pod. *Vanilla planifolia* vanillin synthase (VpVAN) catalyzes the conversion of ferulic acid and ferulic acid glucoside into vanillin and vanillin glucoside, respectively. Desorption electrospray ionization mass spectrometry imaging (DESI-MSI) of vanilla pod sections demonstrates that vanillin glucoside is preferentially localized within the mesocarp and placental laminae whereas vanillin is preferentially localized within the mesocarp. VpVAN is present as the mature form (25 kDa) but, depending on the tissue and isolation procedure, small amounts of the immature unprocessed form (40 kDa) and putative oligomers (50, 75 and 100 kDa) may be observed by immunoblotting using an antibody specific to the C-terminal sequence of VpVAN. The VpVAN protein is localized within chloroplasts and re-differentiated chloroplasts termed phenyloplasts, as monitored during the process of pod development. Isolated chloroplasts were shown to convert [¹⁴C]phenylalanine and [¹⁴C]cinnamic acid into [¹⁴C]vanillin glucoside, indicating that the entire vanillin *de novo* biosynthetic machinery converting phenylalanine to vanillin glucoside is present in the chloroplast.

Keywords: Chloroplasts • Immunolocalization • Phenyloplasts • Vanillin biosynthesis • Vanillin synthase • VpVAN.

Abbreviations: Chl, chlorophyll; d, days; DESI-MESI, desorption electrospray ionization mass spectrometry imaging; DTT, dithiothreitol; FITC, fluorescein isothiocyanate; PAL, phenylalanine ammonia lyase; PBS, phosphate-buffered saline; TCEP, tris(2-carboxyethyl)phosphine; TLC, thin-layer chromatography; UGT, UDP-glucosyltransferase; VpVAN, *Vanilla planifolia* vanillin synthase.

Introduction

In the last several decades, extensive studies have been carried out to gain a better understanding of the cellular machineries involved in efficient and channeled synthesis of plant natural products. One approach towards elucidation of the biosynthetic

pathways involved and the physiological roles of the compounds produced is to determine the tissue, cellular and subcellular localization of their site of biosynthesis and accumulation. In this study, we set out to determine the biosynthetic production site of vanillin (4-hydroxy-3-methoxybenzaldehyde), the world's most popular flavor and aroma compound.

Vanilla and its key flavor component vanillin are universally appreciated flavors. Vanilla is the complete alcohol extract of the mature vanilla seed capsule, commonly called a pod (Sinha et al. 2008). Vanilla plants belong to the *Orchidaceae* family, the genus *Vanilla*, tribe *Vanilleae*, subfamily *Vanilloideae* (Cameron et al. 1999, Cameron 2004, Cameron and Molina 2006). Among the approximately 110 vanilla species in the genus *Vanilla*, three species are used for production of vanilla extracts by local farmers as well as at an industrial scale: *Vanilla planifolia*, *V. tahitensis* and *V. pompona*. *Vanilla planifolia* is the most valued among these three species because of its high vanilla flavor quality, providing 95% of the world vanilla pod production (Odoux and Grisoni 2010).

Vanilla planifolia is a climbing perennial vine with a large, green and succulent stem that is photosynthetic. The plant produces oblong, smooth, bright green leaves, and adventitious aerial roots that grow opposite each leaf, aiding lateral support. The roots are associated with endotrophic mycorrhiza (Anuradha et al. 2013). The vanilla flowers are yellow, bisexual and develop towards the top of the plant when the vine is approximately 4–5 m long. When successful pollination occurs, each flower yields a single pod. The pod reaches its full size about 3 months after pollination. The immature green *V. planifolia* pods are almost odorless as the key flavor components are stored as glucosides. Mature pods are approximately 15 cm long and are pale green to yellow in color (Odoux and Brillouet 2009). *Vanilla planifolia* pods are harvested when they are 8–9-months-old, before the pods begin to dehisce. Freshly harvested pods are processed by curing to stop the natural vegetative processes, to initiate the enzymes responsible for the formation of the aromatic flavor constituents and to prevent microbial growth, thereby enabling long-term preservation (Odoux and Grisoni 2010).

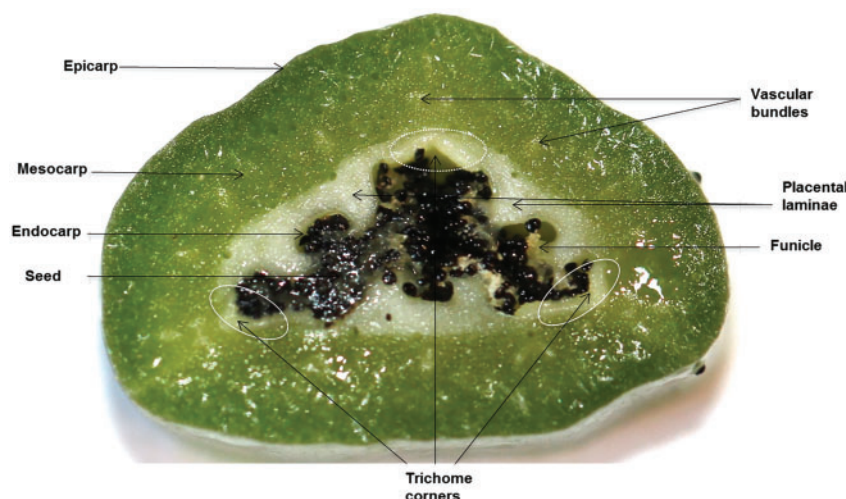


Fig. 1 Transverse section of a 6-month-old vanilla pod, with arrows pointing to the different tissues present.

Fig. 1 shows a transverse section of a *V. planifolia* pod to demonstrate its anatomy and tissue terminology. The *V. planifolia* pod encompasses three areas, which are visually distinct; the outer part (greener area), inner part (white/yellowish green area) and seeds. The outer part includes the epicarp and outer mesocarp. The inner part includes the inner mesocarp, placental laminae, endocarp and seeds. In total, the mesocarp is formed by 15–20 layers of large cells. Seeds are localized towards the cavity of the pod (**Fig. 1**) (Odoux et al. 2003b, Odoux and Brillouet 2009).

The vanilla pod is known to produce >200 different flavor compounds (Sinha et al. 2008). Vanillin is the most abundant compound and provides the key flavor and aroma of the vanilla extract and of the cured *V. planifolia* pod (Sinha et al. 2008). The compound vanillin is suggested to have various physiological functions in the plant (Burri et al. 1989, Lopezmalo et al. 1995). As vanillin is toxic to living organisms in high concentrations (Boonchird and Flegel 1982), *V. planifolia* plants store vanillin as vanillin- β -D-glucoside, a conjugated form with glucose, commonly called vanillin glucoside or glucovanillin.

Vanilla planifolia pods are the prime plant organ source of vanillin and the site of vanillin glucoside biosynthesis and storage (Odoux et al. 2003b, Odoux and Brillouet 2009, Gallage et al. 2014). Vanillin glucoside starts to accumulate in the inner part of the pod when they are 3-month-old, and continues to do so until the pod is 7–8-months-old, reaching concentrations >300 mM in the water phase of the mesocarp cells (Odoux et al. 2003b, Odoux et al. 2006, Odoux and Brillouet 2009, Palama et al. 2009). Vanillin glucoside was shown by Odoux et al. (2003b) to accumulate in the inner part of the mesocarp and placental laminae, as the main sites. In contrast, Havkin-Frenkel and Dixon reported that vanillin glucoside was produced and accumulated in a unique hairy secretory tissue, the trichomes, and accumulated in the secretion around the seeds (Joel et al. 2003). In a subsequent and thorough study, the conclusions of the latter study were refuted (Odoux and Brillouet 2009). Vanillin is distributed in similar tissues to vanillin glucoside yet at an about 20- to 50-fold lower

concentration (Odoux et al. 2003b, Odoux and Brillouet 2009). Vanillin and its glucoside are absent from seeds (Odoux and Brillouet 2009).

Vanillin production in *V. planifolia* may be divided into three modules: synthesis of vanillin via ferulic acid by a C–C chain shortening step, glucosylation of vanillin to vanillin glucoside (the non-toxic storage form) and hydrolysis of vanillin glucoside and liberation of the aromatic compound vanillin.

The biosynthetic pathway of vanillin in the pod of *V. planifolia* has recently been elucidated. Vanillin is synthesized via conversion of ferulic acid and ferulic acid glucoside to vanillin and vanillin glucoside, respectively (Negishi et al. 2009, Gallage et al. 2014). This reaction is catalyzed by a single enzyme, referred to as vanillin synthase (VpVAN, accession No. KP278240.1) as demonstrated by the ability of the enzyme to convert ferulic acid/ferulic acid glucoside into vanillin/vanillin glucoside following coupled transcription/translation of the VpVAN gene in *in vitro* assays, following transient expression of the gene in *Nicotiana benthamiana* and following stable expression in *Hordeum vulgare* and *Saccharomyces cerevisiae* (Gallage et al. 2014).

VpVAN has high amino acid sequence similarity to enzymes of the cysteine protease family (Gallage et al. 2014). Cysteine proteases are a large group of enzymes with versatile physiological functions and not very well defined substrate specificities (Storer and Menard 1996). Papain (EC 3.4.22.2) from the latex of *Carica papaya* is the best studied plant cysteine protease (Otto and Schirmeister 1997, Shindo and Van der Hoorn 2008). In general, cysteine proteases are expressed as immature proteins with an N-terminal endoplasmic reticulum (ER)-targeting signal peptide being part of a propeptide domain comprising 130–160 residues (Wiederanders et al. 2003, Cambra et al. 2012). In the mature protein, the propeptide sequence is removed either by a processing enzyme or by autocatalytic processing (Turk et al. 2012). Two putative protease cleavage sites in VpVAN were predicted at residue 61 (RFAR/RYGK) (Gallage et al. 2014) and residue 135 (VD/GVLPVT) (Yang et al. 2017). The *in vitro* transcription/translation experiments

showed no evidence of autocatalytic processing of the VpVAN protein. This indicates that removal of the propeptide requires the action of a separate processing enzyme (Gallage et al. 2014). The physiological function of the VpVAN propeptide is not known. In general, it has been proposed that the propeptide sequence may control proper intracellular targeting, promote proper folding of the mature enzyme and/or serve to maintain the enzyme in an inactive form in the cell to balance its function according to physiological demands (Turk et al. 2012). In the cysteine protease papain, the presence of the N-terminal propeptide blocks the active site cleft directly by non-covalent interactions (Turk et al. 2012). Both the immature and mature forms of VpVAN were shown to be catalytically active, with the latter being more active (Gallage et al. 2014).

The glucosylation step resulting in the conversion of vanillin into its glucoside is much less defined. It is not known at which step glucose incorporation occurs in the biosynthetic pathway. If vanillin glucoside is preferentially made directly from ferulic acid glucoside, the glucosylation may proceed at the level of *p*-coumaric acid, caffeic acid or ferulic acid. In the green pod, vanillin is almost entirely stored as vanillin glucoside, demonstrating that the glucosylation of vanillin is highly efficient or, alternatively, that free vanillin is not involved as an intermediate in vanillin glucoside formation. Our previous proteomic and transcriptomic studies demonstrated that several UDP-glucosyltransferases (UGTs) are present in the *V. planifolia* pod. Of these, VpUGT72E1 has been shown to be a vanillin-specific glucosyltransferase (Gallage et al. 2014).

The third module in vanillin formation involves hydrolysis of vanillin glucoside and liberation of the aromatic compound. Hydrolysis of vanillin glucoside takes place when the cells are broken down as part of the pod maturation process or as a result of pathogen attack and under the curing process. The enzyme which is involved in hydrolyzing vanillin glucoside, vanillin β -D-glucosidase, has been well characterized (Odoux et al. 2003a, Odoux et al. 2003b). The β -D-glucosidase was purified to homogeneity and demonstrated to be a tetramer (201 kDa) composed of four identical subunits (50 kDa). The optimum pH was 6.5 and the K_m value for vanillin glucoside was 20.0 mM; V_{max} was 5.0 μ kat mg^{-1} (Odoux et al. 2003a). The high K_m value matches the high substrate concentration. At the tissue level, vanillin β -D-glucosidase activity was found to be distributed in the inner mesocarp and placental laminae in a similar manner to vanillin glucoside (Odoux et al. 2003b). The subcellular localization of the β -D-glucosidase throughout pod development was determined using a specific antibody and confocal microscopy (Brillouet et al. 2014), and it was demonstrated that the β -D-glucosidase was localized as in a corona around re-differentiating chloroplasts, probably being situated in the lumen between the inner and outer envelopes. In 4-month-old pods, few re-differentiating chloroplasts were visible among the photosynthetically active chloroplasts in the cytoplasm of cells of the inner mesocarp. In 8-month-old pods, almost all chloroplasts were re-differentiated, as demonstrated by the presence of a corona of β -D-glucosidase and complete loss of Chl red fluorescence. The re-differentiation of chloroplasts was followed by transmission electron microscopy during

pod ontogeny and demonstrated a progressive dismantling of the grana thylakoids and thylakoid budding, resulting in the formation of circular membrane vesicles packed with ribosomes. At full pod maturity, internal membrane structures were no longer visible but the β -D-glucosidase remained localized as a corona around the re-differentiated chloroplasts (Brillouet et al. 2014).

Brillouet et al. also proceeded to investigate the subcellular localization of vanillin glucoside during pod ontogeny (Brillouet et al. 2014). Using multiple cell imaging approaches combining immunohistochemistry localization by confocal microscopy and fluorescence spectral analysis by multiphotonic microscopy, it was discovered that vanillin glucoside was progressively stockpiled in the inner volume of the re-differentiated chloroplasts as solid amorphous deposits. The vanillin glucoside concentration was estimated to exceed 4 M and the deposition as an amorphous solid was suggested to ensure homeostasis. At the end of the re-differentiation process, the vanillin glucoside deposits filled the entire plastidial volume (Brillouet et al. 2014). Based on the discovered function of the re-differentiated chloroplasts as a storage site for a phenolic plant-specialized metabolite, the re-differentiated chloroplasts were named phenyloplasts (Brillouet et al. 2014). In summary, the vanillin glucoside biosynthetic pathway, the storage site of vanillin glucoside in re-differentiated chloroplasts termed phenyloplasts as well as the β -D-glucosidase involved in aroma release have been elucidated.

In our current study, we set out to increase further our understanding of how *V. planifolia* orchestrates the biosynthesis of vanillin glucoside in such high concentrations by investigating the cellular and intracellular site of vanillin biosynthesis in the *V. planifolia* pod and the post-translational processing of VpVAN. Desorption electrospray ionization mass spectrometry imaging (DESI-MSI) was used to visualize the distribution of the main flavor components within the *V. planifolia* pod. A polyclonal antibody specific for the C-terminal sequence of VpVAN was used to demonstrate that the VpVAN protein and thus vanillin glucoside biosynthesis is localized in chloroplasts and in the re-differentiated chloroplasts termed phenyloplasts. Radiolabeling experiments support that chloroplasts *de novo* synthesize vanillin glucoside from phenylalanine and cinnamic acid.

Results

Vanillin glucoside is localized in the inner part of the vanilla pod where vanillin biosynthetic activity is detected

Administration of ^{14}C -radiolabeled precursors to *V. planifolia* pods harvested at 3, 4, 5, 6, 7, 8 and 9-months following pollination demonstrated that the pods actively biosynthesized vanillin. The studies with radiolabeled precursors were carried out using *V. planifolia* pods shipped from La Reunion to Copenhagen by courier mail while still attached to their vine. In one set of experiments, [^{14}C]phenylalanine, [^{14}C]cinnamic acid, *p*-[^{14}C]hydroxybenzaldehyde and [^{14}C]vanillin were

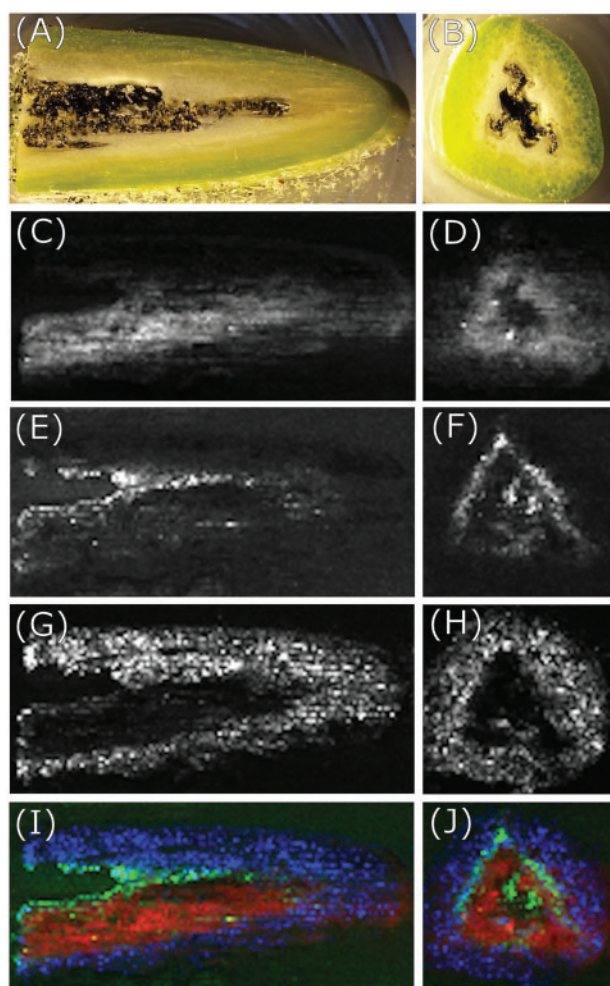


Fig. 2 Images obtained by DESI-MSI of longitudinal (left column) and cross- (right column) sections of a 6-month-old vanilla pod. (A and B) Photo of vanilla pod tissue obtained immediately following longitudinal and cross cryosectioning, respectively. (C and D) Vanillin distribution (m/z 153). (E and F) Vanillin glucoside distribution (m/z 337). (G and H) Sucrose distribution (m/z 381). (I and J) Colored overlay of vanillin (red), vanillin glucoside (green) and sucrose (blue) distributions.

administered to the inner yellow fleshy region of the pod including the inner mesocarp, placental laminae and endocarp. In a second set of parallel experiments, the radiolabeled compounds were administered to the thick dark green outer part of the pod including mainly the epicarp and outer mesocarp. Radiolabeled metabolites formed were separated by thin-layer chromatography (TLC) (**Supplementary Fig. S1**). Upon administration of [^{14}C]phenylalanine and [^{14}C]cinnamic acid to the inner part of the pod, a radiolabeled product co-migrating with the vanillin glucoside authentic standard was observed, while administration of p -[^{14}C]hydroxybenzaldehyde gave rise neither to formation of radiolabeled vanillin nor to vanillin glucoside. These results show that an active vanillin glucoside biosynthetic machinery is situated in the inner part of the *V. planifolia* pod throughout pod development. The time course study also demonstrated that administration of

p -[^{14}C]hydroxybenzaldehyde did not contribute to vanillin biosynthesis at any of the time points examined during *V. planifolia* pod development. It should be noted that the administration of radiolabeled [^{14}C]vanillin resulted in formation of radiolabeled vanillin glucoside in the inner as well as the outer part of the vanilla pod, demonstrating that vanillin glucosyltransferase activity was also present in parts of the pod not catalyzing *de novo* synthesis of vanillin (**Supplementary Fig. S1**).

DESI-MSI was carried out to visualize the localization of the two main metabolites, vanillin and vanillin glucoside, in the *V. planifolia* pod using longitudinal and cross-sections of a 6-month-old *V. planifolia* pod (**Fig. 2**). In the recordings obtained, the color intensity of a pixel in each of the compound-specific images is scaled relative to the intensities for that compound in the rest of the image, and not relative to the signal of other detected compounds. MS images thus provide information about the distributions of individual compounds, but not about their mutual abundances. Comparison of absolute intensities obtained with different compounds in the imaging experiment cannot be translated into mutual abundances due to their differences in ionization efficiency.

As shown in **Supplementary Fig. S1**, radiolabeling experiments based on administration of [^{14}C]radiolabeled precursors demonstrated that vanillin biosynthesis takes place in the inner part of the pod. DESI-MS images (**Fig. 2C, D and E, F**) visualize the tissue distribution of vanillin and vanillin glucoside, respectively, in a 6-month-old pod. Both compounds are present in the tissues where biosynthetic activity was detected. Vanillin glucoside is specifically localized within the inner mesocarp, placental laminae and endocarp (**Fig. 2E, F**), while vanillin is distributed within the mesocarp (**Fig. 2C, D**). The partly non-superimposed distribution of vanillin and vanillin glucoside suggests that one or more vanillin β -glucosidases and/or vanillin glucosyltransferase activity are differentially distributed at this pod developmental stage (**Fig. 2C–F**).

Presence of VpVAN as demonstrated by Western blot analysis of *V. planifolia* protein extracts and following transient expression of VpVAN in tobacco leaves

The presence of the VpVAN protein was monitored during pod development using a specific VpVAN antibody prepared towards the C-terminal peptide (NH_2 -) CNWGDNGYFKMELGK (-CONH₂) and used in a 1:5,000 dilution. No background reactions were observed using pre-immune serum from the same rabbit (**Supplementary Fig. S2**). In agreement with previous results (Gallage et al. 2014), the recognized protein bands at 25 and 40 kDa match the calculated molecular masses of 23.89 and 39.15 kDa of mature and immature VpVAN, respectively. These immunoblot patterns demonstrate that VpVAN undergoes post-translational maturation in *V. planifolia* as well as in tobacco where the propeptide is cleaved off, as generally observed for classical cysteine proteases (Wiederanders et al. 2003, Gallage et al. 2014). Comparing the strength of the band patterns, it is apparent that VpVAN is present almost entirely in its mature form in 7-month-old *V. planifolia* pods (**Fig. 3**).

The highest concentration of vanillin and vanillin glucoside is found in 6- to 7-month-old pods. It is thus likely that the activity of the protein is stimulated upon maturation of VpVAN (Fig. 3).

The formation and post-translational modification of VpVAN was further investigated following *Agrobacterium*-mediated transient expression of the VpVAN-encoding gene in *N. benthamiana* (tobacco). Crude protein extracts of the VpVAN-expressing leaves were obtained 7 d after *Agrobacterium* infiltration and probed with the antibody specific to the C-terminal sequence of VpVAN at a 1:5,000 dilution (Fig. 3). Western blot analyses showed the presence of a dominant immunoreactive band with an apparent mass of 25 kDa representing mature VpVAN, indicating effective processing of VpVAN into the processed mature form after 7 d of transient expression in *N. benthamiana* (Fig. 3).

Mature VpVAN oligomers detected in the crude protein extracts from *V. planifolia* pods as demonstrated by Western blotting analysis

Cysteine proteinases are known to form dimers (Vincent and Brewin 2000), and dimerization has been associated with gain of optimal protease activity (Olsen et al. 2009). The possible occurrence of dimers of VpVAN was investigated by SDS-PAGE fractionation of a protein extract from a 7-month-old *V. planifolia* pod followed by Western blot analysis using a dilution series of the VpVAN C-terminal sequence-specific antibody. When the VpVAN C-terminal antibody was used at a 1:50 dilution, five protein bands migrating with apparent molecular masses of 25, 40, 50, 75 and 100 kDa were detected (Fig. 4). The VpVAN C-terminal antibody did not show cross-reactivity towards any of the major protein components in the crude

protein extract from *V. planifolia* pods (Fig. 4). In agreement with previous results (Gallage et al. 2014), the 25 and 40 kDa bands match the calculated molecular masses of 23.89 and 39.15 kDa of mature and immature VpVAN, respectively, and the 50, 75 and 100 kDa bands possibly represent homodimers, trimers and tetramers of the mature VpVAN. When the Western blot analyses were carried out using antibodies applied at increasingly higher fold dilutions (Supplementary Fig. S3), the 50, 75 and 100 kDa bands vanished, possibly demonstrating that oligomeric forms of mature VpVAN had reduced binding affinity for the antibody compared with the monomer of mature VpVAN (Supplementary Fig. S3).

In attempts to convert the putative oligomers of mature VpVAN into the 25 kDa monomer, the protein extract from an 8-month-old vanilla pod was reduced with tris(2-carboxyethyl)phosphine (TCEP) before SDS-PAGE. The concentrations of TCEP tested varied from 5 to 50 mM. At none of these concentrations did the presence of TCEP result in reduced band intensities at 50, 75 and 100 kDa (Fig. 5; Supplementary Fig. S4). Oxidation of the protein extract with potassium ferricyanide also did not result in intensification of the 50, 75 and 100 kDa bands (Fig. 5). Based on the specificity of the antibody used, we propose that the immunoreactive bands at 50, 75 and 100 kDa represent oligomers of the 25 kDa monomer formed as artifacts during homogenization of the vanilla pod. The inner part of the vanilla pod contains high amounts of oleoresins and a highly viscous mucilaginous material impregnating the proteins upon homogenization of the vanilla pod, as previously reported (Odoux 2005). Random protein cross-linking reactions are thus likely also to result in the formation of oligomers of VpVAN not cleavable by TCEP.

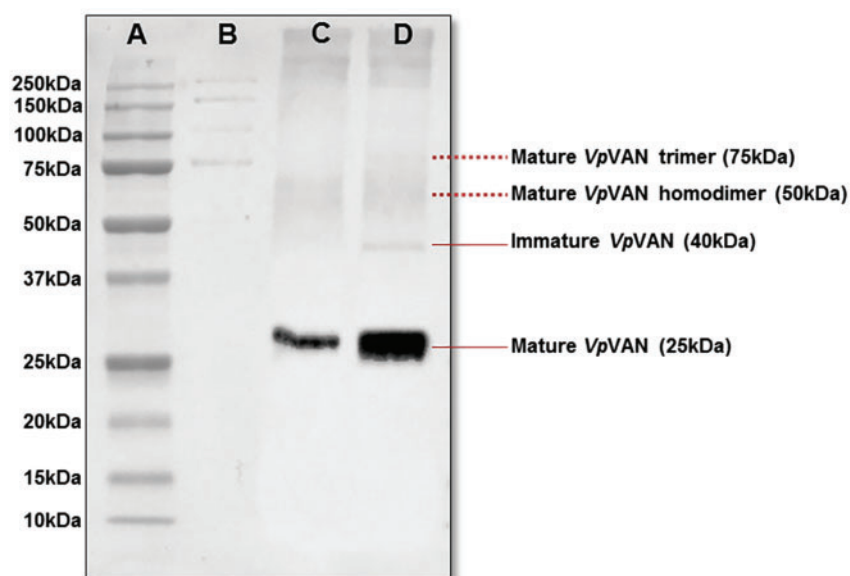


Fig. 3 Western blot analysis of the presence of mature and immature forms of VpVAN in protein extracts of vanilla pods and in *N. benthamiana* leaves following transient expression of VpVAN. Lane A: pre-stained Bio-Rad protein ladder. Lane B: unstained Bio-Rad protein ladder. Lane C: protein extract from *N. benthamiana* leaves following transient expression of VpVAN probed with an antibody specific to the C-terminal sequence of VpVAN. Lane D: protein extract of a 7-month-old vanilla pod probed with an antibody specific to the C-terminal sequence of VpVAN. A 10 µg aliquot of protein were applied to each lane.

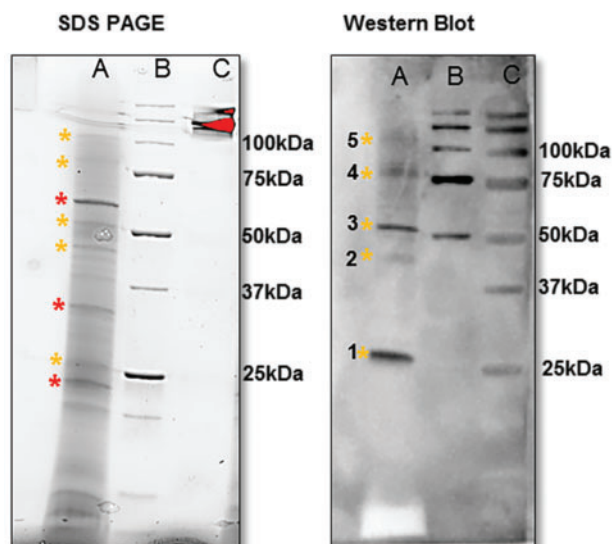


Fig. 4 Mature VpVAN homodimer and putative oligomers detected in the crude *V. planifolia* extracts as demonstrated by SDS–PAGE followed by Western blot analysis. Lane A: crude protein extract from a 7-month-old vanilla pod from *V. planifolia*. Lane B: pre-stained Bio-Rad protein ladder. Lane C: unstained Bio-Rad protein ladder. 1, VpVAN mature protein (25 kDa); 2, VpVAN immature protein (39 kDa); 3, putative mature VpVAN homodimer (50 kDa); 4, putative mature VpVAN trimer (75 kDa); 5, putative mature VpVAN oligomers (100 kDa). The proteins present in a 7-month-old vanilla pod were separated by SDS–PAGE (12% Criterion™ TGX Stain-Free Precast Gels) and visualized using the ChemiDoc MP Imaging System (Bio-Rad). The major protein components present in the pod extracts are marked with * (red) while VpVAN and putative oligomers are marked with * (yellow). Western blot analysis was conducted using a C-terminal sequence-specific antibody against VpVAN in a 1:50 dilution. At this extremely high antibody concentration, the primary antibody demonstrated specificity towards VpVAN. No cross-reactions were observed with the major protein components present in the extract.

To reduce protein impregnation with the mucilaginous material and obtain sharper protein bands upon SDS–PAGE, the vanilla pod protein extracts used for Western blots in the current study were extracted in an optimized buffer composed of 10 mM MES, 2 mM dithiothreitol (DTT) at pH 5 as recommended for protein extraction of complex fruit tissues (Wang et al. 2008).

VpVAN is localized in the chloroplasts and phenyloplasts as demonstrated by immunohistochemical analyses

The tissue and cellular localization of VpVAN in fresh 7-month-old *V. planifolia* pods was examined by confocal microscopy. The immunohistochemical analyses were based on use of the specific antibody to the C-terminal sequence of VpVAN and thus targeted both the immature and the mature form of VpVAN. The images obtained by confocal microscopy clearly demonstrated that the localization of VpVAN was restricted to specific plastids present in multiple copies and situated within the cytoplasmic space of the cell (Fig. 6). Control images were

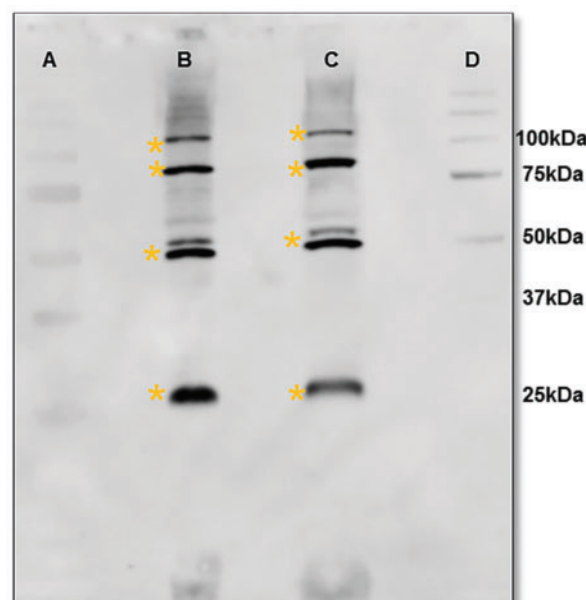


Fig. 5 The ratio between monomeric and putative oligomeric forms of VpVAN as analyzed by treatments with reductant and oxidant as monitored by SDS–PAGE followed by Western blot analysis. Crude protein extracts from 8-month-old pods were analyzed. Protein bands recognized by the VpVAN antibody are marked with * (yellow). Lane A: pre-stained Bio-Rad protein ladder. Lane B: treatment with 50 mM TCEP for 15 min. Lane C: treatment with 50 mM TCEP for 15 min followed by 50 mM potassium fericyanide for 30 min. Lane D: unstained Bio-Rad protein ladder.

obtained using pre-immune serum at the same dilution, and in these experiments no reactions mimicking those of the VpVAN antibody were observed (Supplementary Figs. S5, S6). The instrumental gain setting which gave a minimum fluorescence background when used with the pre-immune serum was also used in the experiments with the VpVAN antibody. The fluorescence signal observed using the VpVAN antibody thus represents specific immune labeling of VpVAN.

Light microscopy of transverse sections of 7-month-old *V. planifolia* pods demonstrated the presence of chloroplasts throughout the entire mesocarp layer (Fig. 7A, D, G). The chloroplasts were likewise detected by their red autofluorescence following excitation at 580 nm (Fig. 7C, F, I). The plastids present in the cytosolic matrix labeled by the specific VpVAN antibody were identified as chloroplasts in light microscopy- and fluorescence microscopy-based studies in which the chloroplasts were identified visually based on their Chl content and red autofluorescence in parallel with simultaneous superimposed immunohistochemical localization of VpVAN (Fig. 7A, D, and C, F).

It is evident, that some of the antibody-labeled plastids did not show the red autofluorescence characteristic of chloroplasts or only weak autofluorescence (Fig. 7B, E, and C, F). These plastids have the same overall dimensions as the chloroplasts. Independent of the Chl content and level of red autofluorescence, these plastids gave a positive

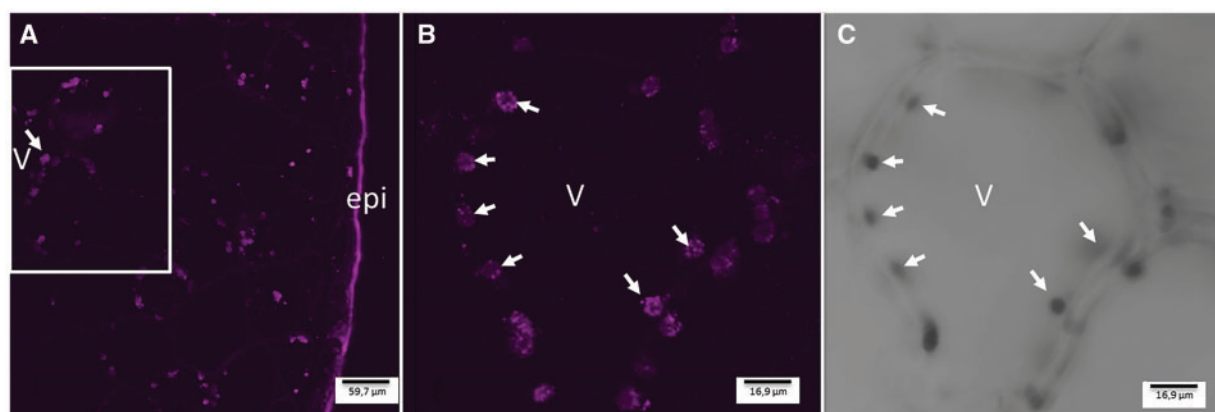


Fig. 6 Immunohistochemical localization of VpVAN in cytoplasmic organelles in a 7-month-old *V. planifolia* pod as observed by confocal microscopy. (A) Transverse section of a 7-month-old *V. planifolia* pod providing an overview of VpVAN localization in the mesocarp. (B) Close-up of a single mesocarp cell showing the intracellular localization of VpVAN in plastids in the cytoplasm. (C) The same section as shown in (B) observed with translucent light. Images were obtained from transverse section of a 7-month-old *V. planifolia* pod using the VpVAN C-terminal antibody and a goat anti-rabbit antibody labeled with FITC. The images were recorded using a Leica SP1 confocal scanning microscope. Arrows indicate the position of selected cytoplasmic plastids harboring VpVAN. Abbreviations: epi, epicarp; v, vacuole.

immunochemical reaction with the specific antibody towards VpVAN. This indicated that VpVAN is localized in chloroplasts as well as in cytoplasmic-localized plastids that are devoid of Chl or with reduced Chl content. Re-differentiating chloroplasts have previously been reported to be present in vanilla pods and demonstrated to be the main storage sites of vanillin glucoside. The re-differentiated chloroplasts are referred to using the term phenyloplasts and are devoid of Chl (Brillouet *et al.* 2014). In the merged images (**Fig. 7C, F**), the chloroplasts harboring VpVAN are observed as orange (**Fig. 7C, F**, white arrows) whereas VpVAN-containing phenyloplasts completely devoid of Chl are observed as green (**Fig. 7C, F**, white stars). With the fluorescence filter settings used to record fluorescein isothiocyanate (FITC) labeling of the immunolocalized VpVAN as well as auto-fluorescence of chloroplasts, it is very likely that chloroplasts in the transition of being converted into phenyloplasts are observed as having a yellowish color due to reduced Chl content.

The localization of VpVAN was followed at 1 month intervals throughout pod development (3–9 months). Throughout the entire development phase, VpVAN was observed to locate strictly to chloroplasts and phenyloplasts (**Supplementary Fig. S7**). In fully mature 9-month-old pods, the signals became weak and diffuse (data not shown).

Agrobacterium-mediated transient expression of the gene encoding VpVAN in *N. benthamiana* leaves resulted in what would appear to represent a similar subcellular localization of VpVAN in chloroplasts (**Supplementary Fig. S8**). However, using this experimental system, strong background fluorescence was observed from the plant cell walls. This strong background fluorescence was also observed in control experiments in the absence of the antibody targeting the C-terminal sequence of VpVAN. The use of this analytical system for localization of VpVAN was therefore considered not to provide unambiguous conclusions and was not further pursued.

VpVAN detection and functional activity in isolated chloroplasts from *V. planifolia*

The demonstrated localization of VpVAN in chloroplasts prompted us to isolate intact chloroplasts from 8-month-old *V. planifolia* pods to test their biosynthetic activity. The isolated chloroplast preparations were examined by light microscopy to ascertain their purity and intactness (**Fig. 8A**). The chloroplasts were isolated following gentle homogenization of the pod tissue in 50 mM HEPES (pH 8.0)/0.33 M sorbitol and purified using Percoll gradients. Radiolabeled precursors were administered to the isolated intact chloroplasts. Following incubation for 24 h, the radiolabeled products formed were separated by TLC. The isolated chloroplasts were functionally active, as demonstrated by the production of radiolabeled metabolites upon administration of [14 C]phenylalanine, [14 C]cinnamic acid and [14 C]vanillin (**Fig. 8**). Administration of [14 C]phenylalanine resulted in formation of [14 C]cinnamic acid (**Fig. 8, 1B**). This conversion is catalyzed by phenylalanine ammonia lyase (PAL), and PAL is known to be localized in the chloroplasts (Sainders and McClure 1975). [14 C]Vanillin was readily converted to [14 C]vanillin glucoside, demonstrating the presence of vanillin glucosyltransferase activity (**Fig. 8, 3B**). Furthermore, a radiolabeled product co-migrating with [14 C]vanillin glucoside was observed upon administration of [14 C]phenylalanine and [14 C]cinnamic acid (**Fig. 8, 1B** and 2B). As expected, the strongest labeling of [14 C]vanillin glucoside was observed upon administration of [14 C]cinnamic acid, a more specific vanillin glucoside precursor compared with phenylalanine.

The presence of VpVAN in protein extracts obtained from the intact chloroplasts isolated from 8-month-old *V. planifolia* pods was analyzed by SDS-PAGE and Western blot experiments (**Supplementary Figs. S9, S10**). A specific antibody towards PSI subunit D (PSI-D) (Haldrup *et al.* 2003) was used as a positive control (**Supplementary Fig. S9**). PSI-D is an 18 kDa subunit of the PSI complex and localized on the stromal side of

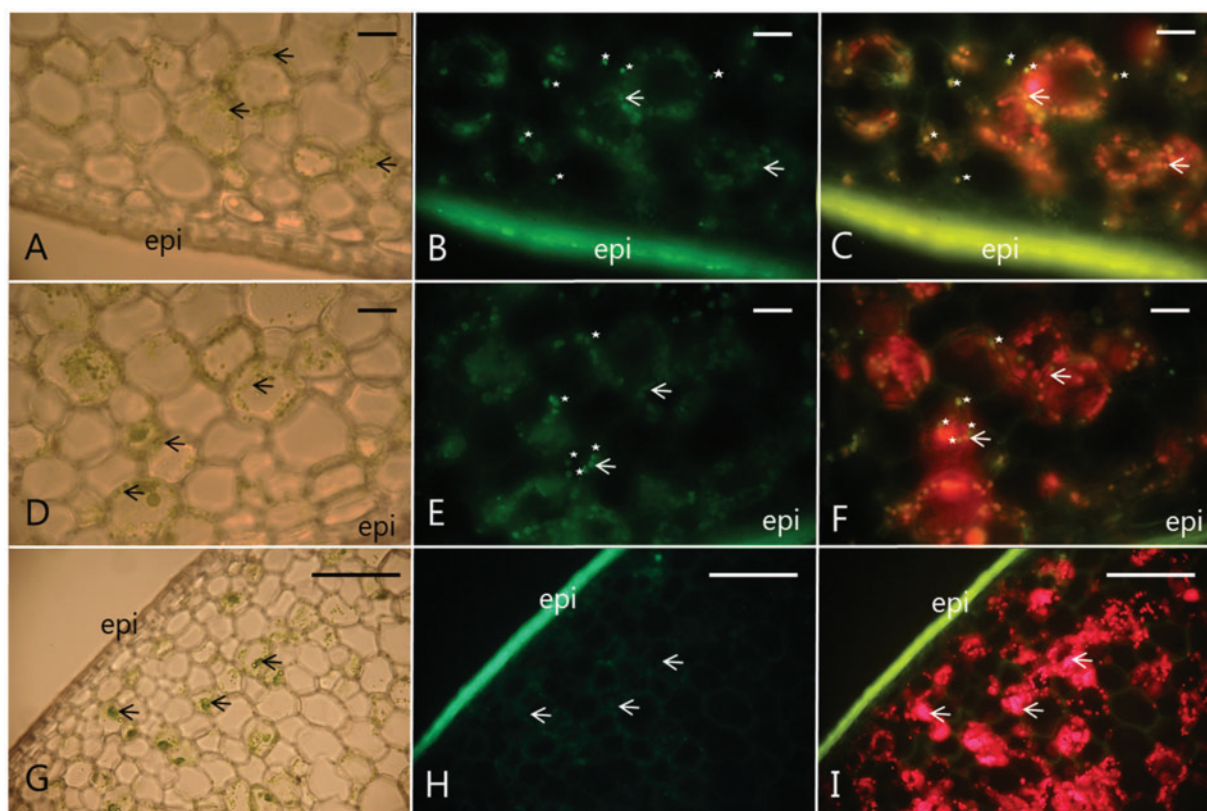


Fig. 7 Immunolocalization of VpVAN to chloroplasts and phenyloplasts in 7-month-old *V. planifolia* pods using an antibody specific to the C-terminal sequence of VpVAN. (A–F) Data obtained from two transverse sections of two different pods are shown; *V. planifolia* pod 1 (A–C) and *V. planifolia* pod 2 (D–F). (A, D and G) Visualization of the examined transverse vanilla pod sections using light microscopy documenting the localization of chloroplasts. Chloroplasts are identified based on their Chl content. Selected chloroplasts are marked with black arrows. (B and E) Immunodetection of VpVAN in chloroplasts and phenyloplasts by FITC and fluorescence microscopy. The chloroplasts and phenyloplasts are stained green due to the antibody reaction. Selected phenyloplasts and chloroplasts are marked with white stars and white arrows, respectively. (C and F) Localization of VpVAN in chloroplasts (white arrows) and phenyloplasts (white stars) as monitored by fluorescence microscopy using a filter setting enabling simultaneous detection of FITC and Chl fluorescence. Chl fluorescence appears in red and demonstrates the localization of chloroplasts. Phenyloplasts that are totally free of Chl appear in green on (C) and (F) while those plastids retaining a small amount of Chl due to incomplete re-differentiation appear in yellow in (C) and (F). (G, H and I) Control panels; vanilla pod sections probed with pre-immune serum. No cross-reactions were observed. Chloroplasts: black arrows (A, D and G) and white arrows (B, C, E, F, H and I). Phenyloplasts: white stars. The same selected set of chloroplasts and phenyloplasts is labeled on the different panels to facilitate interpretation of the nature of the observed structures. Scale bars correspond to 100 μ m. epi, epicarp.

the PSI complex in the thylakoid membrane. In most experiments, immunoreactive bands at 25 and 40 kDa corresponding to the mature and immature forms of monomeric VpVAN were not observed using the antibody specific to the C-terminal sequence of VpVAN. Instead a strong immunoreaction was observed in the 50 kDa region as also observed on the Western blots with crude *V. planifolia* pod protein extracts when VpVAN C-terminal antibody was used in a dilution of 1:300 (**Supplementary Fig. S10**). Thus, in the isolated chloroplasts, the 25 kDa VpVAN monomer was converted into its oligomeric form. Oligomerization may have been favored by the extended procedure and the high pH of the isolation buffer required for isolation of intact chloroplasts. The isolation of intact chloroplasts from the vanilla pods was hampered by the high amounts of mucilaginous material and oleoresins present in the pods.

Discussion

When the current study was initiated, the enzyme VpVAN catalyzing vanillin glucoside synthesis from ferulic acid and the β -glucosidase catalyzing liberation of free vanillin had been identified. Likewise, re-differentiated chloroplasts named phenyloplasts had been shown to be the storage site of vanillin glucoside. In the present study, we demonstrate that the chloroplasts and phenyloplasts are also the site of accumulation of VpVAN. In agreement with those observations, tracer studies further support that isolated chloroplasts are able to synthesize vanillin glucoside *de novo*.

The VpVAN-catalyzed synthesis of vanillin glucoside from ferulic acid glucoside proceeds as a retro aldol elimination reaction resulting in C3 side chain shortening of ferulic acid glucoside (Gallage et al. 2014). Such a pathway was initially

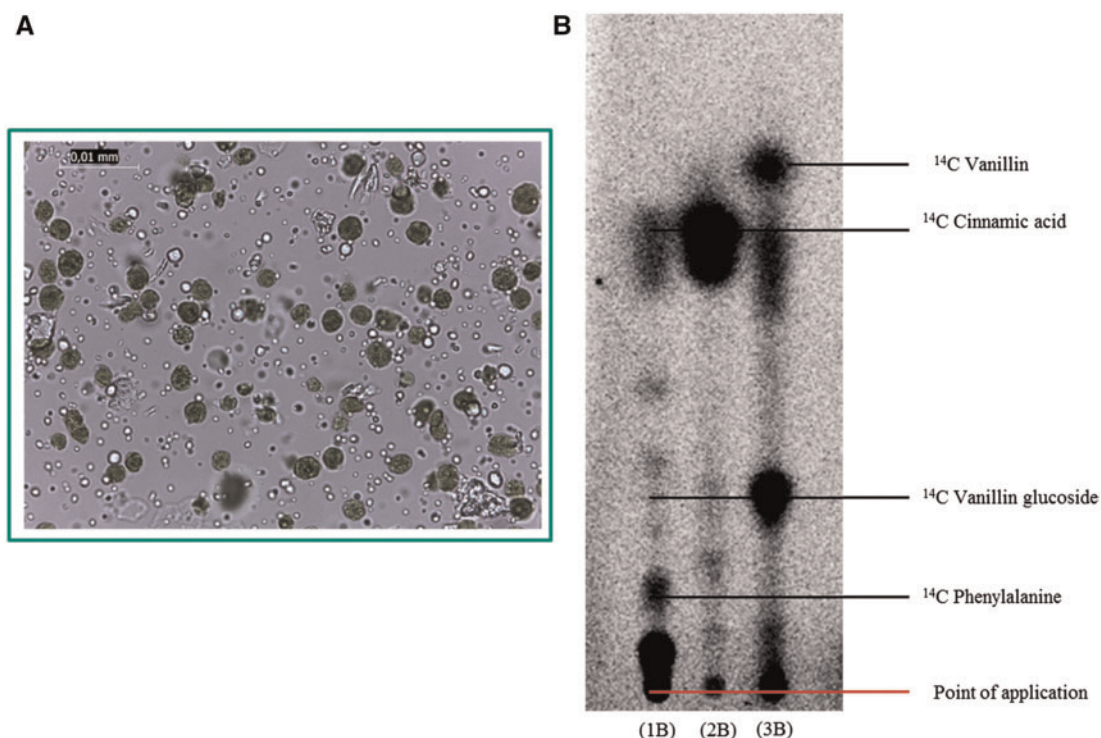


Fig. 8 Intact chloroplasts isolated from 8-month-old *V. planifolia* pods synthesize vanillin *de novo*. (A) The purity and integrity of the isolated chloroplasts as monitored by light microscopy. (B) Vanillin biosynthetic activity in isolated chloroplasts as monitored by administration of [^{14}C]phenylalanine (lane 1B) and [^{14}C]cinnamic acid (lane 2B) as substrates. An extract from the inner part of a *V. planifolia* pod disc incubated with [^{14}C]vanillin was applied (lane 3B) to visualize the migration position of [^{14}C]vanillin glucoside.

proposed by Zenk (1965) and subsequently by Negishi et al. (2009) based on radioactive precursor studies using [^{14}C]ferulic acid. In the present study, radiolabeling studies confirmed that active vanillin glucoside biosynthesis takes place throughout pod development (3–9 months) and that *p*-hydroxybenzaldehyde did not serve as a vanillin precursor.

DESI-MSI requires minimal sample preparation and thereby minimizes contamination and formation of artifactual compounds. MSI is emerging as an excellent alternative to classical analytical methods where breakdown of plant constituents may occur in the course of preparation (Bjarnholt et al. 2014). In the present study, DESI-MSI showed localization of vanillin and vanillin glucoside in the inner part of the pod, where vanillin glucoside biosynthetic activity likewise was detected by radioactive precursor administration. The concentration of vanillin is about 20- to 50-fold lower than that of vanillin glucoside (Odoux 2005) and their distribution patterns are not superimposable. This indicates that β -glucosidase activities involved in vanillin glucoside hydrolysis or glucosyltransferase activities catalyzing its formation varies across the placental laminae and mesocarp. A radial distribution of vanillin glucoside and the β -glucosidase enzyme activity has previously been reported (Odoux et al. 2003b). The radiolabeling experiments based on administration of [^{14}C]vanillin indeed showed the presence of glucosyltransferase activity in both the inner and outer parts of the pod. The localization in the *V. planifolia* pod of vanillin-specific glucosyltransferases has not been thoroughly studied.

To determine the subcellular localization of the vanillin biosynthetic machinery, we investigated the cellular and intracellular localization of the key enzyme of vanillin biosynthesis, VpVAN. Immunolocalization studies clearly demonstrated that VpVAN was localized in chloroplasts distributed within the inner mesocarp and placental laminae of the vanilla pod throughout *V. planifolia* pod development (3–9 months). Furthermore, studies of VpVAN demonstrated that the protein underwent a maturation step involving removal of the propeptide sequence corresponding to mobility shifts on SDS–PAGE from 40 to 25 kDa positions. We previously reported that removal of the propeptide sequence augmented the activity of VpVAN (Gallage et al. 2014). Putative dimers, trimers and tetramers of mature VpVAN were observed in crude protein extracts of the vanilla pods and especially in isolated intact chloroplasts. Self-association of proteins forming dimers or oligomers is a common phenomenon. Some cysteine proteases such as caspases are known to undergo dimerization as a requirement to gain catalytic activity and enzyme activation (Grzonka et al. 2001, Marianayagam et al. 2004, Mackenzie and Clark, 2012). In the present study, the oligomeric forms could not be converted into the monomeric form by treatment with a strong reductant such as TCEP. Thus, we conclude that these oligomers are artifacts formed as a result of a chemical reaction of the VpVAN monomer with the abundant mucilaginous material present in the pods.

Administration of [^{14}C]phenylalanine to intact chloroplasts isolated from the vanilla pod resulted in formation of a

radiolabeled product co-migrating with [^{14}C]vanillin glucoside, suggesting that the entire vanillin biosynthetic machinery responsible for conversion of phenylalanine to vanillin glucoside operated in the chloroplasts. It is not known at which stage in the vanillin pathway the glucosylation takes place. If vanillin glucoside is preferentially made directly from ferulic acid glucoside, the glucosylation may proceed at the level of *p*-coumaric acid, caffeic acid or ferulic acid. The storage form of vanillin in the *V. planifolia* pod is the vanillin β -D-glucoside. High concentrations of vanillin are toxic to the cell (Boonchird and Flegel 1982). Accordingly, a vanillin-specific UGT would be expected to be co-localized with VpVAN to catalyze conversion of any vanillin formed into vanillin β -D-glucoside to avoid cell toxicity. A study has shown that UDP-glucose, which is the co-substrate for family 1 glucosyltransferases, is de novo synthesized in the chloroplasts of *A. thaliana* (Okazaki et al. 2009).

The classes of plant natural products that are synthesized in the chloroplasts are remarkably diverse. In addition to photosynthesis, chloroplasts are known to carry out many other essential functions such as synthesis of amino acids (Kirk and Leech 1972), fatty acids (Lippold et al. 2012), lipids (Wang and Benning 2012), plant hormones (Metraux 2002) and vitamins (Dellapenna and Pogson 2006). Most of these compounds are not only essential for chloroplasts to accomplish their metabolic role but at different stages of plant ontogeny are released from the chloroplast to serve directly as or be metabolized into signaling components for plant growth and development or as defense compounds against pathogens or herbivores (Joyard et al. 2009). Synthesis of aromatic amino acids in the chloroplasts would constantly provide phenylalanine as the initial substrate for vanillin biosynthesis. The phenylalanine precursors chorismic acid and shikimic acid are known to form esters with *p*-coumaric acid, which are intermediates in ferulic acid biosynthesis. In 1966, conversion of *p*-coumaric acid into caffeic acid was demonstrated in isolated chloroplasts from leaves of *Saxifraga stolonifera*, which shows 4-hydroxycinnamoyl transferase (4-HCL) activity in the chloroplasts (Satô 1966, Bassard et al. 2012).

While the chloroplast genome encodes about 80–100 proteins, between 2,500 and 3,500 nuclear-encoded proteins are predicted to be targeted to the chloroplast (Joyard et al. 2009). In general, chloroplast proteins encoded by the nuclear genome and imported into chloroplasts are synthesized as precursor proteins with cleavable N-terminal transit peptides that direct each protein to its final destination within the chloroplast subcompartments (Nielsen et al. 1994, Bruce 2000). Sequence analysis of the vanillin biosynthetic pathway genes (Gallage et al. 2014) encoding VpVAN and putative *V. planifolia* PAL, putative VpC4H (cinnamate-4-hydroxylase), putative Vp4-HCL, putative VpHCT (hydroxycinnamoyl transferase), putative VpC3H (cinnamoyl ester 3' hydroxylase), putative VpCOMT (caffeic acid *O*-methyl transferase) and putative VpUGT using the chloroplast transit peptide prediction program SignalP (<http://www.cbs.dtu.dk/services/SignalP/>) did not provide evidence for the presence of a cleavable transit peptides within the encoded proteins. However, the function of chloroplasts as an organelle harboring entire pathways for secondary metabolites

is far from being understood, and further biochemical evidence may serve to upgrade the prediction software (Kießling et al. 2000, McAndrew et al. 2001).

Phenyloplasts have been shown to be the storage site of vanillin glucoside (Brillouet et al. 2014). The phenyloplasts arise following re-differentiation of chloroplasts resulting in loss of their Chl content and photosynthetic abilities, and gain of the ability to store high concentrations of phenylpropanoid-derived glucosides (Brillouet et al. 2013, Brillouet et al. 2014). Re-differentiation of chloroplasts to different storage plastids is a well-known phenomenon (Weier 1936, Leyon 1953, Kutik 1998). Leucoplasts are colorless plastids that function as storage organelles. Leucoplasts comprise amyloplasts, oleoplasts and proteinoplasts, and these are known to store starch, lipids and proteins, respectively. In fleshy fruits such as tomatoes, ripening is associated with the re-differentiation of green fruit chloroplasts into ripe fruit chromoplasts (Klee and Giovannoni 2011, Llorente et al. 2016, Llorente et al. 2017). Chromoplasts contain carotenoid pigments that give the red, orange and yellow colors to the plant structure (Roberts 1946, Egea et al. 2010, Camara et al. 1982, Llorente et al. 2017). Young chromoplasts are metabolically active but contain fewer DNA copies than chloroplasts. Gerontoplasts are known to be the last ontogeny stage of chloroplasts, and these plastids no longer harbor functional DNA (Sitte 1977, Kutik 1998).

In analogy to the re-differentiation processes of chloroplasts described above resulting in formation of plastids accumulating primary metabolites (amyloplasts, oleoplasts and proteoplasts) as well as secondary metabolites in the form of pigments (chromoplasts), it is not surprising that re-differentiated chloroplasts may also store secondary metabolites as phenylpropanoids. Strong scientific documentation for this was first provided using the vanilla pod as the experimental system. Brillouet et al. (2014) coined the term phenyloplasts for this type of re-differentiated chloroplasts. The grana thylakoids of the vanilla chloroplasts were dismantled in the early phases of phenyloplast ontogeny, and no deposits of phenolics were observed within the thylakoidal lumen. Based on indirect histochemical data, phenyloplasts have earlier been suggested as storage sites for secondary metabolites (Saunders and McClure 1976, Zaprometov and Nikolaeva 2003, Liu et al. 2009). Thus, the storage of vanillin glucoside in phenyloplasts may not represent a unique case of subcellular sequestration of phenolics in the plant kingdom. Some secondary metabolites accumulate in massive amounts in certain plant tissues. This applies to the cyanogenic glucoside dhurrin which in the tip of etiolated seedlings of *Sorghum bicolor* (L.) constitutes up to 30% of the dry weight (Saunders and Conn 1977, Halkier and Møller 1989), and flavan-3-ols which in leaves of *Camellia sinensis* (L.) constitute up to 30% of the dry weight (Liu et al. 2009). Such high concentrations of secondary metabolites might be attractive to store in phenyloplasts (Gachon et al. 2005).

Biological research concerning the *V. planifolia* orchid, vanilla pods and the physiological role of vanillin formation has been paid relatively little attention by the research community in spite of the importance of the vanilla flavor (Gallage and Møller 2015). Production of vanilla from the vanilla orchid is

highly labor intensive and a lengthy process not easily adapted to market demands. Global demand for vanilla was estimated at between 2,500 and 3,000 Mt annually in 1998, while the global demand for vanillin was estimated at around 16,000 Mt annually in 2010 and worth US\$ 650 million in total on the world market (Smolarski 2012). However, only 0.25% of vanillin originates from cured pods of the vanilla orchid, *V. planifolia* (Gallage and Møller, 2015). Today 99% of all vanillin consumed worldwide is synthetically made, primarily using chemical synthesis based on petrochemicals, or chemically derived by acid hydrolysis of lignin. Market pull and consumer demand have promoted intensive research to develop sustainable biological production platforms for vanillin using microorganisms as a replacement for environmentally unsustainable chemical synthesis (Gallage and Møller 2015).

The demonstration that vanillin biosynthesis takes place in the chloroplasts would open the door for design of photosynthetic production platforms using algae, cyanobacteria or moss producing vanillin. When fully developed, such production platforms offer superior alternatives to classical biotechnological hosts such as bacteria and yeast for vanillin biosynthesis because they use carbon dioxide as the sole carbon source and sunlight as the energy source. Production in photosynthetic organisms such as cyanobacteria offers a sustainable alternative, because the carbon skeletons, energy and reducing power are derived from photosynthesis via CO₂ fixation and light-driven electron transport. Using the cyanogenic glucoside dhurrin as a model system, such photosynthetic systems are now also being developed for synthesis of complex diterpenoids (Møller 2014, Lassen *et al.* 2014, Gnanasekaran *et al.* 2016, Møller 2017).

In a recent report including Hailian Yang, Daphna Havkin-Frenkel and Richard A. Dixon as authors (Yang *et al.* 2017), the catalytic activity of VpVAN was re-evaluated. It was concluded that the VpVAN protein catalyzes conversion of *p*-coumaric acid to *p*-hydroxybenzaldehyde or as an alternative that this conversion proceeds non-enzymatically (Yang *et al.* 2017). In a previous publication, we studied the catalytic activity of VpVAN in *in vitro* and *in vivo* experiments and documented that the VpVAN enzyme catalyzes the conversion of ferulic acid and ferulic acid glucoside to vanillin and vanillin glucoside, respectively (Gallage *et al.* 2014). This result has also been obtained in patent applications (Havkin-Frenkel *et al.* 2006, Havkin-Frenkel and Podstolski 2007, Gallage *et al.* 2014). The catalytic property of VpVAN as a vanillin synthase has been verified in independent studies demonstrating vanillin and vanillin glucoside formation in *Capsicum frutescens* (hot chili pepper) stably transformed with VpVAN (Chee *et al.* 2017), and further substantiated in the present study.

In the previous studies by Daphna Havkin-Frenkel and Richard A. Dixon, a 28 kDa protein was partially purified from an embryo culture extract of *V. planifolia* and used for preparation of an antibody. As already pointed out by Odoux *et al.* (2009), this antibody was not raised towards a single purified protein but based on a protein fraction with the ability to convert *p*-coumaric acid into *p*-hydroxybenzaldehyde. In the recent publication by Yang *et al.* (2017), the 28 kDa protein reported to be encoded by the same gene sequence as

VpVAN was isolated from a crude *V. planifolia* embryo culture extract following immune purification using the antibody described above followed by elution of the protein at pH 2.6. The eluted and supposedly reconstituted protein was reported to convert *p*-coumaric acid to *p*-hydroxybenzaldehyde as monitored by HPLC analysis. No activity was obtained with ferulic acid as a substrate, in contrast to what was observed in previous studies (Havkin-Frenkel *et al.* 2006, Havkin-Frenkel and Podstolski 2007, Gallage *et al.* 2014, Chee *et al.* 2017). In our study, we obtained the native immature and mature VpVAN protein using a rabbit reticulocyte lysate-based transcription/translation system (Gallage *et al.* 2014). The mature and immature proteins migrated on SDS-PAGE with apparent molecular masses of 25 and 40 kDa, respectively. Using highly sensitive LC-MS (ion trap) analyses, VpVAN was demonstrated *in vitro* to catalyze the conversion of ferulic acid into vanillin. No production of *p*-hydroxybenzaldehyde could be detected using *p*-coumaric acid as substrate. These results have been confirmed and further substantiated in the current study.

In our previous publication on VpVAN (Gallage *et al.* 2014), *in vivo* studies were carried out by transient expression of the VpVAN-encoding gene in *N. benthamiana* and by stable expression of the gene in yeast and barley. In all these studies, a codon-optimized gene sequence was used to achieve efficient gene expression. Expression of the transgene was in all cases correlated with formation of vanillin or vanillin-derived metabolites, whereas formation of *p*-hydroxybenzaldehyde or metabolites thereof was not correlated with transgene expression. The studies reported by Yang and co-workers were carried out without codon optimizations. Heterologous expression of VpVAN in host systems such as yeast requires prior modification of endogenous background reactions in the host system to achieve production of vanillin glucoside. Guidance on how to achieve this in combination with sensitive analytics based on use of LC-MS ion trap instrumentation to monitor the activity of VpVAN was provided in our previous publication (Gallage *et al.* 2014).

In conclusion, in this study, we demonstrated the tissue and intracellular localization of the vanillin biosynthetic machinery in chloroplasts and re-differentiated chloroplasts termed phenyloplasts during pod development. Vanillin biosynthetic activity was demonstrated in isolated chloroplasts, and VpVAN was identified by immunolocalization in both chloroplasts and phenyloplasts, exactly the plastids that have previously been documented to store vanillin glucoside. Furthermore, VpVAN expression studies highlighted that VpVAN indeed undergoes a maturation step where the propeptide is cleaved off. With these results, we are one step closer to understanding how *V. planifolia* is able to produce vanillin in high concentrations during the 5–6 month period during which it develops from an immature to a mature pod.

Materials and Methods

Biological materials

Healthy vines of *V. planifolia* carrying foliage and green vanilla pods were harvested at different time points after pollination and were shipped from the

Biological Resource Center (BRC), VATEL, CIRAD, Saint-Pierre, La Réunion, France, by courier carrier to Denmark while maintaining high humidity conditions.

Three-week-old *N. benthamiana* plants were used for the transient expression of the gene encoding VpVAN.

Agrobacterium strain

Agrobacterium tumefaciens strain AGL1 was used for transient and stable expression assays *in planta* and grown following standard procedures in LB medium with appropriate antibiotics (Bach et al. 2014).

Polyclonal antibody

A polyclonal antibody was obtained by immunizing rabbits with the VpVAN-specific peptide sequence (NH₂-)CNWGDNGYFKMELGK(-CONH₂) derived from the C-terminal amino acid sequence of VpVAN (amino acids 327–340) (Agrisera AB, <http://agrisera.com>). To test the specificity of the antibody, crude protein extracts from 7-month-old pods of *V. planifolia* were separated by SDS-PAGE and subjected to Western blotting.

The specificity of the C-terminal VpVAN-specific antibody was investigated using a series of antibody dilutions (1:50, 1:100, 1:500 and 1:1,000). When used in a 1:50 dilution, the antibody reacted with proteins migrating with apparent molecular masses of 25, 40, 50 and 100 kDa. In different series of experiments, the crude protein extract was either reduced by incubating with TCEP (5 to 50 mM, pH 3.5) for 10 min or oxidized by incubating with potassium ferricyanide (10–50 mM, pH 2.95) for 30 min before SDS-PAGE analysis to investigate the presence or formation of protein oligomers. Pre-immune serum from the rabbit used for the antibody production showed no cross-reaction to protein extracts from *V. planifolia* (Supplementary Fig. S2).

Vector constructs

Plant expression vectors for transient expression in tobacco were constructed using Gateway® cloning technology (Life Technologies) as previously described (Gallage et al. 2014).

Biosynthetic assays with green *V. planifolia* pods and isolated intact chloroplasts

The *V. planifolia* pods harvested at 3, 4, 5, 6, 7, 8 and 9 months following pollination were cut transversely into 1–2 mm thick discs (approximately 25 mg FW) using a scalpel and further dissected to separate the inner and outer part of the pod. Radiolabeled precursors (0.5 µCi) were administered to samples of the inner and outer part of the pod and incubated (30 °C) in 400 mM Tris-HCl pH 8, 20 mM MgCl₂ for 6–48 h.

The ¹⁴C-labeled products formed in the experiments with fresh *V. planifolia* pods were extracted by 25% MeOH and applied to Silica Gel 60 F254 TLC plates (Merck, <http://www.merck-chemicals.com>). The plates were developed in ethyl acetate:acetone:dichloromethane:methanol:water (40:30:20:10:8, by vol.), dried, exposed (48 h) on phosphor-imaging screens (Molecular Dynamics, <http://www.molecular-dynamics.com>) and the radiolabeled products visualized using a Storm 860 Molecular Imager (Molecular Dynamics). Identification of the radiolabeled compounds formed was guided by co-application of authentic standards.

The same experimental procedure was followed using isolated intact chloroplasts (10 µl, 0.5 µg Chl µl⁻¹) obtained from 8-month-old *V. planifolia* pods.

Total protein extraction and Western blot analysis

Vanilla planifolia pods were ground in liquid nitrogen with a mortar and pestle. The resulting powder was homogenized in 100 µl of SDS running buffer (Laemmli buffer, Bio-Rad) and 300 µl of 10 mM MES buffer (pH 5–6) including 20 mM DTT. The homogenate was clarified by centrifugation (10,000 ×g, 10 min, 4 °C). Crude protein extracts were incubated at 65 °C for 30 min before being subjected to separation by SDS-PAGE (225 V, 30 min, 400 mA, 300 W) using 12% Criterion™ TGX Stain-Free Precast Gels (Bio-Rad) and Precision Plus Protein Stained and Unstained Standards (Bio-Rad) as molecular mass markers. Western blots of SDS-PAGE-separated proteins were performed

using a Trans-Blot R Turbo Transfer Blotting instrument (Bio-Rad) and Trans-Blot R Turbo Midi Nitrocellulose membranes (Bio-Rad). Membranes were blocked with 5% (w/v) skimmed milk in PBS-T buffer (phosphate buffered saline with Tween-20) for 2 h. The VpVAN C-terminal sequence-specific antibody was applied in a dilution of 1:5,000. The presence of immunoreactive polypeptides was visualized using a horseradish peroxidase-conjugated goat anti-rabbit antibody (DakoCytomations, <http://www.dako.com/dk>) in a 1:5,000 dilution using the Super Signal West Dura extended duration substrate kit (Pierce). In the experiments with pre-sera, the same 1:5,000 dilution of pre-sera and horseradish peroxidase-conjugated goat anti-rabbit antibody was used as advised by the producer (Agrisera).

Blots were developed for 1–30 min with a ChemiDoc MP Imaging System (Bio-Rad) equipped with a cooled CCD camera (Bio-Rad) set to automatic exposure setting. Total protein was visualized on the membranes using the stain-free blot setting. The Precision Plus Protein unstained ladder (Bio-Rad) did not give visible bands below 50 kDa; therefore, the Precision Plus Protein Stained ladder was included in the gel. After blotting, the Precision Plus Protein Stained ladder can be seen with the naked eye. When imaging with the ChemiDoc MP Imaging System, a blot picture was taken before the settings were changed to visualize chemiluminescence. Both blot pictures were then merged to enable visualization of the entire Precision Plus Protein Stained ladder.

The same procedure was followed to extract total protein and to analyze the presence of VpVAN following transient expression of the VpVAN gene in tobacco.

Immunolocalization of immature and mature VpVAN in *V. planifolia* and *N. benthamiana*

To localize both the immature and mature forms of VpVAN, immunolocalization was performed as previously reported (Sanchez-Perez et al. 2012). Infiltrated tobacco leaves or *V. planifolia* pods at different ontogenies were cut into 1 cm pieces, embedded in 5% agarose and cut into 120 µm sections. The sections were placed in 5% skimmed milk in PBS at room temperature to block unspecific background. After 30 min incubation, the C-terminal sequence-specific VpVAN antibody was added at a 1:100 dilution (Kannangara et al. 2011). Following incubation for 2 h, the sections were washed three times with PBS. Secondary antibody (goat anti-rabbit with the FITC fluorophore) was added 1:160 in PBS and incubated for 2 h at room temperature. Thereafter, the sections were washed three times in PBS and left in PBS.

Sections were mounted with anti-fading agent and analyzed by confocal scanning microscopy using Leica SP1 at 488 nm excitation for FITC detection or with a fluorescence microscope, Leica DMR using filters for specific observation of FITC and a combined filter for simultaneous detection of FITC and chloroplast autofluorescence.

Chloroplast isolation and fractionation

Intact chloroplasts were isolated from four 8-month-old *V. planifolia* pods essentially as previously reported (Robinson 2002). Pods were cut transversely into 2 cm long sections and homogenized in HS buffer [50 mM HEPES, KOH (pH 8) and 0.33 M sorbitol]. The homogenate was filtered through nylon mesh (44 µm) and centrifuged (3,330 ×g, 2 min). The chloroplast pellet was gently re-suspended in HS buffer, layered onto pre-cooled Percoll pads and centrifuged (1,400 ×g, 8 min). The intact chloroplast sediment was washed in HS buffer, re-sedimented (3,000 ×g, 2 min) and re-suspended in 50 µl of HS buffer. The purity and integrity of the purified chloroplasts were checked under a Leica ICC50 HD microscope.

Immunoblot analysis of isolated chloroplasts

Samples (40 µl) were heated at 65 °C for 30 min before separation by SDS-PAGE using 12% Criterion TGX Stain-Free pre-cast gels (Bio-Rad) and a Tris/glycine/SDS running buffer (Bio-Rad) at 240 V for 35 min. Proteins were transferred to a 0.2 µm PVDF (polyvinylidene fluoride) membrane using the Trans-Blot Turbo transfer system (Bio-Rad) according to the manufacturer's protocol. The membrane was blocked for 20 min at room temperature with 5% (w/v) skimmed milk in PBS-T buffer and incubated for 1 h with the C-terminal sequence-specific VpVAN primary antibody at a 1:300 dilution in 2% (w/v) skimmed milk in

PBS-T. The blot was washed with PBS-T buffer and incubated with a secondary swine anti-rabbit horseradish peroxidase-conjugated antibody (Dako) using a 1:5,000 dilution in PBS-T for 1 h at room temperature. The membrane was washed again with PBS-T buffer and the secondary antibody detected using SuperSignal West Dura Chemiluminescent Substrate (Pierce) and developed for 1–10 min with a ChemiDoc MP Imaging System (Bio-Rad). A specific antibody to the PSI-D (Haldrup et al. 2003) in a 1:10,000 dilution was used as a reference in quality tests of the isolated chloroplasts.

Desorption electrospray ionization mass spectrometry imaging (DESI-MSI)

Pieces of frozen *V. planifolia* pods were mounted on a cryo-microtome sample holder using water as the only adhesive. Using a Leica CM3050S cryo-microtome (Leica Microsystems), the tissue was cut into 40 µm thin sections, which were thaw-mounted on microscope glass slides and stored at –80°C until the time of analysis. Compared with most other plant tissues, the tissue was very fragile and difficult to section, and therefore a relatively high thickness was chosen. On the day of analysis, the sample slide was taken directly from the freezer to a vacuum desiccator for 10 min prior to DESI analysis.

Imaging was performed on a Thermo LTQ XL linear ion trap mass spectrometer (Thermo Scientific) equipped with a custom-built DESI ion source based on a motorized microscope stage by Märzhäuser Wetzla and controlled by in-house software. The imaging stage is described in detail elsewhere (Thunig et al. 2011). Imaging was performed in the positive ion mode using a 4 µl min^{–1} flow rate of methanol and water (80:20) containing 50 mM NaCl and 0.1% formic acid for enhanced generation of sodium adducts and protonated species. The nebulizer gas pressure was 6 bar. The pixel size was 250 µm. Vanillin was imaged at *m/z* 153 in its protonated state, vanillin glucoside was imaged at *m/z* 337 as its sodium adduct and sucrose (or an isomer) was imaged at *m/z* 381 as its potassium adduct. The raw data files were converted to imzML files (Schramm et al. 2012), and Data Cube Explorer (AMOLF, Amsterdam) was used to generate images. MATLAB was used to create colored overlaid images.

Supplementary Data

Supplementary data are available at PCP online.

Author Contributions

N.J.G. planned and designed the project and performed the ¹⁴C-radiolabeled precursor feeding experiments, TLC analysis, all the molecular biology analysis, tobacco expression studies, protein extractions and expression studies, and contributed to writing the manuscript. K.J. contributed in planning and carrying out all immunolocalization studies, designing the project and writing the manuscript. C.J. carried out the DESI analysis. A.J.Z.N. carried out isolation of intact chloroplasts. T.N. performed vanilla/tobacco expression studies. E.D. performed and optimized vanilla crude protein extraction and Western blotting analysis. L.D. carried out the vanilla pod cryo-sectioning. M.G. was in charge of vanilla pod sampling and courier shipments of fresh vanilla materials from La Reunion to Denmark. B.L.M. planned and designed the project, provided biochemical expertise and scientific mentoring, and contributed to writing the manuscript.

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Disclosures

The authors have no conflicts of interest to declare.

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